EXPERIMENTAL STUDIES OF THE NASOPHARYNGEAL SECRETIONS FROM INFLUENZA PATIENTS.

VIII. Further Observations on the Cultural and Morphological Characters of Bacterium pneumosintes.

By PETER K. OLITSKY, M.D., and FREDERICK L. GATES, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plate 67.

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In an earlier paper of this series[1] *Bacterium pneumosintes*, derived from the nasopharyngeal washings of patients in the early hours of acute epidemic influenza, was described as a minute bacilloid body of regular form, with a length about two to three times its breadth, measuring 0.15 to 0.3 micron in the long axis. It was stated that although longer individuals were seen occasionally, "the organisms showed little tendency to pleomorphism and were characterized by uniformity in size and shape."

At that time, *Bacterium pneumosintes* had been cultivated only in a tissue medium composed of human ascitic fluid and fresh rabbit kidney, sometimes with the addition of beef infusion broth and nutrient agar. The combination of ascitic fluid and fresh kidney tissue has remained the medium of choice for the maintenance of the cultures, because in it they require transfer only at long intervals and they retain their morphological and cultural characteristics through many generations. But during cultivation over a period of 1 to 3 years the three strains[2] at present available have become saprophytic, so that at present they are cultivable anaerobically in a variety of media which are less difficult to prepare. Coincident with this adaptation to a new environment, certain variations in morphology and a loss of pathogenicity for rabbits have been observed. It is the

[1] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 713.

[2] These strains were derived from Cases 16, 17, and 26 (Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 125).

purpose of this paper to describe the cultural and morphological characteristics of *Bacterium pneumosintes* after prolonged artificial cultivation. The three strains have behaved in an identical manner, and a common description will suffice.

Methods of Cultivation.

Even after cultivation for 3 years *in vitro Bacterium pneumosintes* fails to grow in autoclaved media without the nutritive or growth-stimulating factors found in fresh tissue or body fluids. But the ascitic fluid of the original medium may be replaced by peptone broth; and fresh defibrinated blood, vegetable tissue,[3] or the products of bacterial metabolism may be substituted for rabbit kidney. The addition of dextrose (1 per cent) to the medium hastens the establishment of anaerobic conditions, and, judging from the rapidity and density of the growth, increases its nutrient value.

The basis of the media now in use is a 1 per cent peptone beef infusion broth, with sodium chloride 0.5 per cent, titrated to pH = 7.4 on the Sörensen scale. According to the object for which the culture is prepared, this broth is enriched with fresh blood, animal or vegetable tissue, or by the growth of other organisms, as will be described. Solid and semisolid media are made by the addition of nutrient agar in the proper proportion.

When a large inoculum from an enriched medium is added to dextrose broth alone, *Bacterium pneumosintes* grows in the primary, but not in subsequent transplants, showing that the initial growth is dependent upon factors carried over from the tissue medium. Thjötta and Avery[4] have shown how minute is the quantity of the growth-stimulating factors required in the case of Pfeiffer's bacillus. Their V factor in dilutions of 10^{-3} and their X factor in dilutions of 10^{-6} suffice to promote growth. With *Bacterium pneumosintes* the

[3] Dr. O. T. Avery and Dr. H. J. Morgan, at the Hospital of The Rockefeller Institute, found that *Bacterium pneumosintes* grows readily in a simple infusion broth containing a fragment of fresh vegetable tissue (potato, turnip, parsnip, etc.) and kindly permit us to report their observation in advance of their own publication. They described this medium in the *Proceedings of the Society for Experimental Biology and Medicine*, 1921, xix, 113.

[4] Thjötta, T., and Avery, O. T., *J. Exp. Med.*, 1921, xxxiv, 97.

essential growth-stimulating factors appear to be of the same order of efficacy. For example, 20 mg. of fresh rabbit kidney supports growth in 5 cc. of medium. The faint haze of *Bacillus coli* which develops in the 1st hour after inoculation makes dextrose broth a highly favorable medium for *Bacterium pneumosintes*. This interesting symbiotic relationship will be discussed in a subsequent section.

Cultural Characteristics.

Bacterium pneumosintes was described as an obligate anaerobe and has maintained that character. We have successfully cultivated the organism only under a vaseline seal or in the depths of the medium in the presence of active reducing agents such as dextrose, fresh animal or vegetable tissue, or aerobic organisms, or in a strictly anaerobic jar. Under such conditions, under a vaseline seal for example, *Bacterium pneumosintes* grows in enriched dextrose broth in a diffuse cloud throughout the fluid medium. In previously incubated tubes, in which anaerobic conditions are already established, this cloud becomes visible within a few (8 to 16) hours after inoculation and reaches its greatest density in 3 to 5 days, when the culture is opaque by reflected light and shows a smoky translucency by transmitted light. Growth is then checked by acid production. The cloud is too finely divided to show a bacterial shimmer, and remains in suspension for days, gradually settling in an even, amorphous, cream-colored layer in the bottom of the tube. Spontaneous flocculation has not been observed.

In the depths of solid media submicroscopic colonies develop as tiny gray specks, which, under the high power of the microscope, are found to be dense irregular masses of bacteria with a fringe of single organisms.

Dextrose is split by *Bacterium pneumosintes* with acid formation but without gas production. In dextrose broth the limiting hydrogen ion concentration is the same for all three strains; namely, 5.2 to 5.3 on the Sörensen scale. After growth has apparently ceased, however, the organisms remain viable for several days in the acid medium.

Recently the simple, safe, and efficient anaerobic jar described by Brown[5] has furnished us with the conditions necessary for the

[5] Brown, J. H., *J. Exp. Med.*, 1921, xxxiii, 677.

development of surface colonies of *Bacterium pneumosintes*. The jar is a modification of those devised by McIntosh and Fildes[6] and by Smillie,[7] and utilizes the catalytic activity of palladinized asbestos heated by a resistance coil in a wire-screened chamber (the principle of the Davy safety lamp) for the union of the oxygen with hydrogen. In this jar we have used plates and slants of nutrient agar, made with beef infusion broth without dextrose, enriched with 5 per cent of fresh defibrinated rabbit blood.

After incubation for 7 to 10 days blood agar plates sown with *Bacterium pneumosintes* show many very minute colonies, almost submicroscopic in size, which are round, raised, and convex, with an entire edge and a colorless translucency. No characteristic structure has been observed, and the growth does not discolor or precipitate the medium. On account of their minute size the colonies are usually discrete, even when close together, but they coalesce to form raised plaques of confluent growth in the most crowded areas.

These colonies interest us as the first example of which we are aware of surface colony formation by a filter-passing obligate anaerobe. The plates also give us a ready means of purifying contaminated cultures, and are useful for the demonstration of organisms in sparse growths of early generations in the ascitic fluid-kidney medium in which the microscopic observation of *Bacterium pneumosintes* is difficult on account of its minute size and the presence of stained protein precipitate.

Morphology.

The substitution of dextrose-peptone broth for ascitic fluid or serum in the medium results in a considerable change in the morphology of *Bacterium pneumosintes*. The dextrose, by the prompt establishment of anaerobic conditions and by its nutritive value to the organisms, supports a much more luxuriant growth than is found in ascitic fluid media. The bacteria are found in diplo form, or in chains of several members, and many of the individual organisms have increased in length so as to be obviously bacillary. They are plump rods with rather pointed ends, which give them a spindle

[6] McIntosh, J., and Fildes, P., *Lancet*, 1916, i, 768.

[7] Smillie, W. G., *J. Exp. Med.*, 1917, xxvi, 59.

shape. Stains color them deeply only in the middle and fade out towards the ends. In chains the spaces between the members are sharply demarcated. More variation occurs in length than in thickness. Forms from 0.5 to 1.0 micron long are not uncommon in cultures which show the characteristic minute forms also. Figs. 1 and 2 show the relative size and shape of *Bacterium pneumosintes* in a collodion sac dialysate of ascitic fluid and in dextrose broth respectively. A strain which has grown as fusiform bacilli in dextrose broth for several generations reverts to the original minute form on cultivation in a dialysate of the original ascitic fluid medium (Fig. 3). Aside from the differences in length and the greater tendency to chain formation, no irregularities in morphology have been noted in comparison with the early generations.

In films from surface colonies grown for 7 days on rabbit blood agar in an anaerobic jar, most of the organisms are of the minute form, but here also longer individuals are found.

As stated previously, *Bacterium pneumosintes* decolorizes by Gram's method. The organisms stain with the usual basic dyes. They are not motile. Neither capsules nor flagella have been demonstrated.

Serological Reactions.

The fusiform, bacillary forms of *Bacterium pneumosintes* grown anaerobically in enriched dextrose broth media and on blood agar plates have been tested at intervals with the immune rabbit sera produced a year ago with cultures of the organism grown in the collodion sac dialysate of an ascitic fluid-rabbit kidney medium.[8] All three strains are promptly agglutinated by these sera, and, on the other hand, they show no tendency to spontaneous flocculation or to agglutination in normal serum. Their genetic relationship to the original minute forms of *Bacterium pneumosintes* is without question, and is further evidenced by their strictly anaerobic character and their reversion to the minute forms on transfer to the original medium.

Symbiosis.

Mention has already been made of the symbiotic development of *Bacterium pneumosintes* with *Bacillus pfeifferi*, the pneumococcus,

[8] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1922, xxxv, 553.

Streptococcus hæmolyticus and *viridans*, and staphylococci, in cultures accidentally contaminated with these organisms.[1] In a number of instances *Bacterium pneumosintes* was recovered from these mixed cultures by filtration. Since the medium then in use was sufficient for the independent growth of any of these organisms, the coincident development of *Bacterium pneumosintes* and the contaminating organism did not give evidence of a nutritive interchange between them.

More recently, in cultures intentionally inoculated with a strain of *Bacillus mesentericus*, we have observed the growth of *Bacterium pneumosintes* in autoclaved dextrose broth in the absence of a fresh tissue fragment. This observation has led to the development of a simple method of anaerobic cultivation. Aerated dextrose broth is inoculated with a young culture of *Bacillus mesentericus* in which spores have not yet formed. During the first hours after inoculation the organism grows diffusely in a faint cloud without pellicle formation. The reducing activity of this obligate aerobe is shown by the decolorization of methylene blue in such a culture within a few minutes after its introduction. The culture is sealed with vaseline when inoculated, or shortly afterward and growth is inhibited by the exhaustion of free oxygen from the medium. Meanwhile the hydrogen ion concentration of the medium falls from 7.4 to 7.2–6.8. 6.8 appears to be the limiting acidity for our strain of *Bacillus mesentericus*. If such a preparation is then inoculated with *Bacterium pneumosintes*, the anaerobe grows luxuriantly and clouds the medium heavily in the course of 1 or 2 days.

This rapid growth of *Bacterium pneumosintes* carries the hydrogen ion concentration of the medium to 5.3–5.2 in the course of 3 or 4 days. Coincidently the vegetative forms of *Bacillus mesentericus* are killed and undergo autolysis so that finally microscopic films and plate cultures may show only the anaerobic organisms. Usually, however, some few spores of *Bacillus mesentericus* are introduced in the original inoculum or are formed in the culture. These may develop subsequently and complicate results. We have therefore tested a number of other organisms for their nutritive or growth-promoting properties, combined with other characters which make them suitable for use. Of these, *Bacillus coli communis* seems well adapted to our purpose. It multiplies rapidly in plain or dextrose

broth under a vaseline seal, so that a sufficient growth is obtained within an hour or two after inoculation. During this short incubation the hydrogen ion concentration of the medium is only slightly affected. The organism may then be killed by exposure to 100°C. for 15 to 30 minutes without destroying the peculiar nutritive substances it imparts to the medium. At the same time anaerobic conditions are immediately established under the vaseline seal. *Bacillus coli* is practically non-pathogenic, and its autolyzed products are not toxic in the minute amounts contained in these cultures. The *Bacillus coli* broth is inexpensive and simple to prepare, and less liable to contamination than are unheated media containing fresh tissue or blood. It contains a minimum of foreign protein. Finally, it supports an abundant growth of *Bacterium pneumosintes* in successive generations. A large inoculum, 0.2 to 0.5 cc. of fluid culture, should be used for seeding.

Preservation of Stock Cultures.

For the maintenance of our stock cultures we have heretofore relied upon the original ascitic fluid-tissue medium, in which *Bacterium pneumosintes* after a preliminary incubation of 5 to 7 days has remained viable at room temperature for $2\frac{1}{2}$ years. Recently blood broth, without dextrose, has been used for the same purpose with encouraging results. The tests are incomplete and the limit of viability in this medium is not yet known, but *Bacterium pneumosintes* established in artificial culture appears to be a resistant organism, and probably requires transfer only at long intervals. We have also employed the method described by Swift[9] for the preservation of microorganisms by freezing and drying *in vacuo*. *Bacterium pneumosintes* withstands this process. The dried cultures are viable for at least 2 months.

In conclusion, we have at present available a number of cultural methods and culture media suitable for a variety of purposes.

For primary isolations from filtered nasopharyngeal secretions, or from the lung tissues of affected animals, the Smith-Noguchi ascitic fluid-rabbit kidney medium probably insures success in the largest percentage of cases. It was in this medium that the original

[9] Swift, H. F., *J. Exp. Med.*, 1921, xxxiii, 69.

isolations were made and the morphological and cultural characteristics of *Bacterium pneumosintes* were first observed.

For the maintenance of stock cultures also, the ascitic fluid-fresh tissue medium is preferred. Blood broth probably may be substituted in the case of well established cultures. *Bacterium pneumosintes* withstands freezing and drying *in vacuo* and probably is viable for long periods in the dry state.

For the demonstration of sparse growths of the microorganisms in the tissue medium, blood agar plates in an anaerobic jar have advantages over the microscope. They are useful also for the purification of contaminated cultures.

Finally, for immunization and for serological reactions requiring considerable quantities of bacteria with a minimal amount of foreign protein, dextrose broth cultures, enriched by the growth of another microorganism, furnish the most useful product. Suitable suspensions may be obtained also from blood agar plates or from growths in the dialysate liquid of collodion sacs containing the culture medium.

These methods of cultivation are being used in studies of the nasopharyngeal secretions of influenza patients obtained during the present recurrence of influenza in New York City. The results of these studies will be reported in subsequent papers of this series.

SUMMARY.

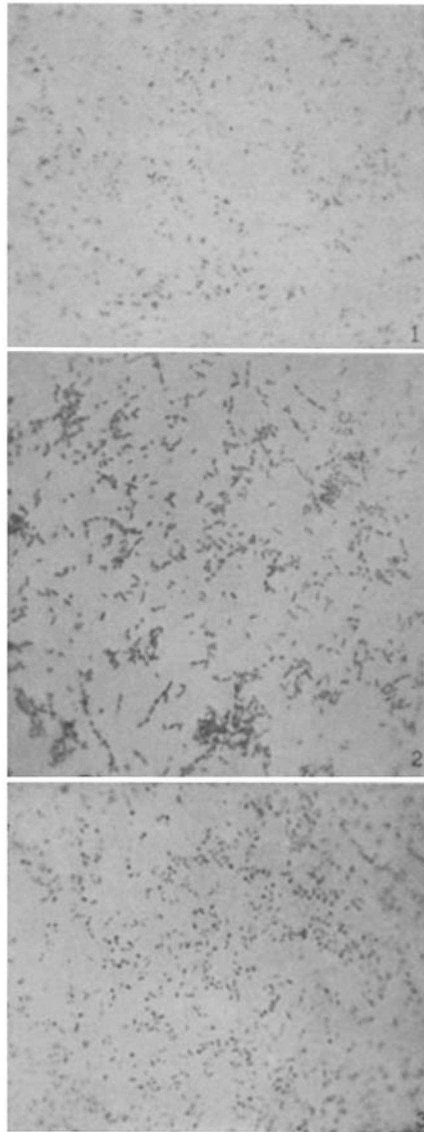
After artificial cultivation for a period of over 3 years *Bacterium pneumosintes* has maintained its original morphological and cultural characteristics, when grown in the original medium. Adaptation to a saprophytic existence has been accompanied by a loss of pathogenicity. Our strains now grow readily under strictly anaerobic conditions in a variety of media with peptone broth as a base, enriched with fresh tissue, blood, or by the growth of other bacteria. Surface colonies have been obtained on blood agar plates in an anaerobic jar. These various methods of cultivation are adapted to special purposes. In broth cultures *Bacterium pneumosintes* grows in larger forms than in the ascitic fluid-tissue medium, but the identity of the microorganisms is proved by their serological reactions and by reversion to the minute forms on transfer to the original medium.

EXPLANATION OF PLATE 67.

FIG. 1. *Bacterium pneumosintes*, Strain 16, in the twenty-fourth generation. Originally isolated March 30, 1919. Film from a collodion sac dialysate of ascitic fluid-rabbit kidney medium. × 1,000.

FIG. 2. *Bacterium pneumosintes*, same strain. Film from a culture in dextrose broth and rabbit kidney medium. × 1,000.

FIG. 3. *Bacterium pneumosintes*, same strain. Film from a collodion sac dialysate of ascitic fluid-rabbit kidney medium seeded from a culture in dextrose broth and rabbit kidney. Note the reversion to the original form. × 1,000.



(Olitsky and Gates: Nasopharyngeal secretions from influenza. VIII.)